Morphine, which is commonly used for the treatment of severe pain, is metabolized essentially in the liver (Pacifici et al., 1982), gastrointestinal tract, kidney, and brain in rodents and humans (Del Villar et al., 1974; Horton et al., 1991; Wahlstrom et al., 1988; Yue et al., 1988). The main metabolic pathways include glucuronidation to morphine 3-glucuronide (M3G)\(^1\) and morphine 6-glucuronide (M6G), N-demethylation to normorphine, and sulfonation to morphine 3- and 6-sulfate (Evans and Shanahan, 1995; Oguri et al., 1970; Yeh et al., 1977). After morphine administration, plasma glucuronides circulate at higher concentrations than morphine (Frances et al., 1992; Säwe et al., 1985). Morphine glucuronides may interact with the opioid receptors and thus contribute to the pharmacological and/or toxicological effects of morphine. Though M3G exhibits no analgesic effects after microinjection into the periaqueductal gray matter or after systemic administration (Gong et al., 1991; Pasternak et al., 1987), M6G has been demonstrated to be a much more potent analgesic agent than morphine when injected iv, it, icv, or sc into mice or rats (Frances et al., 1992; Pasternak et al., 1987; Stain et al., 1995). In man, M6G has demonstrated interesting analgesic properties when iv-injected (Osborne et al., 1992). However, no information has been available concerning its stability and especially its hydrolysis into morphine after oral administration. Initial degradation of M6G could occur in the stomach via acidic hydrolysis before reaching the gut. In the intestinal tract, M6G may be hydrolyzed by β-glucuronidase, a cytosolic enzyme in intestinal mucosal cells (Koster et al., 1985) and also present in intestinal bacteria, and subsequently reabsorbed as morphine. The activity of β-glucuronidase in intestinal cells is low in the duodenum and jejunum and higher throughout the terminal ileum, colon, and rectum (Koster et al., 1985). Nevertheless, anaerobes have been demonstrated to be probably responsible for most of the β-glucuronidase activity in both the small and large intestine (Hawksworth et al., 1971; Walsh and Levine, 1975). Intestinal hydrolysis of glucuronide is dependent on the bacterial population in the gut, which is higher in the terminal intestine and feces than in the small intestine (Walsh and Levine, 1975). In duodenum, M6G could be absorbed into the systemic circulation before reaching the liver and thus undergo hepatic metabolism or biliary excretion. The only available information concerns the other glucuronide conjugate, M3G, which appears following metabolism of morphine by UDP-glucuronyl transferase and is excreted via the biliary canal after undergoing enterohepatic recirculation (EHR) (Walsh and Levine, 1975). Fifty percent of the oral dose of morphine is excreted as M3G in the bile, 20% is found as morphine in the feces, and at least 30% of M6G dose is reabsorbed from the intestinal tract (Walsh and Levine, 1975). The final site of morphine and glucuronide elimination is the kidney, which has been shown to be the most important site for the elimination of M3G and probably M6G (Van Crugten et al., 1991).

Because of these multiple physiological sites involved in M6G disposition, assessment of its hydrolysis into morphine is of interest. The rat is a suitable model to study the pharmacokinetics of M6G because morphine is not metabolized to M6G in the rat (Aasmundstad et al., 1993; Coughtrie et al., 1989). First, we determined the hydrolysis of M6G in an acidic medium to reflect the acid pH in the
stomach; then we investigated the action of β-glucuronidase on M6G contained in different parts of the intestinal lumen. Hepatic effects were studied using the IPRL, which also allows determination of biliary excretion. Finally, the plasma concentration and urinary excretion of M6G, M3G, and morphine vs. time were investigated following M6G administration by the oral route.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 250–300 g (Ifla Credo, Lyon, France) were used. For the plasma and urine kinetics study, rats were placed in metabolic cages, which allowed collection of urine by natural voiding during the whole experiment. All rats had free access to standard laboratory chow and water.

Chemicals and Reagents. M6G and morphine was obtained from Franco-pia-Sanofi (Paris). The purity of M6G (C12H12NO5·2H2O; molecular weight = 497.5) after receipt was checked by HPLC with dual fluorimetric-diode array detection (see below for HPLC procedure). Purity was ≥98.5%. The drug was stable for at least 24 hr at pH ranging from 3.0 to 7.0; no morphine peak was detected. M6G was dissolved in saline just before use. M3G (molecular weight = 461.5) was purchased from Sigma (St Quentin Fallavier, France). β-Glucuronidase was originated from limpets type LII (Patella vulgata) (ref. GB1832, Sigma, France) and was conserved at laboratory temperature. Other chemicals were of HPLC grade and purchased from Sigma (France) or Merck (Nogent sur Marne, France).

Acidic Hydrolysis. Acidic hydrolysis of M6G was performed in 5 ml of saline containing M6G (2.01 μmol/liter) and by adding different amounts of concentrated 1 N HCl to adjust the pH from 1 to 5 with a pH meter (Hanna instrument 8417). The solution was incubated at 37°C in a water bath for 30 min or 1 hr. The hydrolysis reaction was stopped by pH adjustment to pH 7.0 with 0.1 N NaOH.

Action of Fecal Enzymes on M6G. A preliminary experiment was conducted on one rat. After decapsulation, the intestinal content was collected and suspended in medium consisting of 0.5 g of glucose, 0.5 g of peptone, and 0.5 g of yeast dissolved in 100 ml of phosphate solution (pH 7.4). The suspension was centrifuged at 1500 rpm for 2 min. A solution of M6G (to obtain a final concentration of 2.01 μmol/liter) was added to 1 ml of the supernatant. The final suspension was aliquoted, incubated under anaerobic conditions, and stopped at 0.3, 0.5, 1.0, 2.0, 14, 15, 17, and 23 hr by addition of 1 ml of acetonitrile. Morphine concentrations in 100-μl samples were determined by HPLC.

Finally, a group of four rats was killed by decapsulation. Colonic and small intestinal feces were collected and suspended in separate preparations for each rat. A solution of M6G (to obtain a final concentration of 2.01 μmol/liter) was added to 1 ml of the supernatant, and the final suspension was incubated for 23 hr. Morphine concentrations were determined by HPLC for each fecal suspension. M6G hydrolysis in a NaCl buffer (pH 5.2, 37°C) with β-glucuronidase (10,000 units/ml), which are optimal conditions (Combie et al., 1982), was performed as control. Morphine formation was expressed as the per cent of the initial M6G concentration.

Isolated Perfused Rat Liver. Four rat livers were isolated and perfused as described by Bazin-Redureau et al. (1995) with some modifications. Ether-anesthetized rat was given heparin (100 units) via the penile vein. The liver was exposed and the bile duct cannulated with PE-10 tubing (Biotrol, Paris). The portal vein was cannulated with PE-200 tubing, the liver was transferred to a thermostatically controlled Plexiglas chamber (37°C), and a cannula attached to the perfusion system was secured in the portal vein. The outflow of the perfusate from the vena cava was collected in a reservoir. The perfusate (120 ml) consisted of 40 ml of rat donor blood, 80 ml of 4.5% bovine serum albumin in Krebs-Ringer bicarbonate solution (pH 7.4) to give a hematocrit of 13%. Per fusate oxygenated with O2/CO2 (95.5%) was recirculated at a mean flow rate of 50 ml/min with a Masterflex pump (Bioblock, Paris) over a 3-hr period. A solution of 0.5 M NaHCO3, 3 mM sodium taurocholate, and 5 g/liter glucose was continuously infused into the reservoir at a flow rate of 0.015 ml/min to maintain pH of the perfusate at 7.4. Temperature and pH of the perfusate, portal vein pressure, and bile flow were continuously monitored. biochemical controls of liver viability were performed in the erythrocyte-free perfusate (pH 7.4) with a centrifugal analyzer and consisted of measurement of glucose (Gluco-quant kit, Boehringer, Meylan, France), LDH (Enzyme LDH/HBDH kit, BioMérieux, St Marcy l’étoule, France), and electrolites (Na+, K+)(Ciba Corning flame photometer).

The liver was allowed to equilibrate for 1 hr, during which viability controls were performed before injection of M6G (2.01 μmol). Bile was collected in preweighed vials at 0–15, 15–30, 30–45, 45–60, 60–90, 90–120, and 120–180 min. 1-ml perfusate samples were collected at different intervals after M6G injection. Bile samples were stored at −20°C until analysis. M6G, morphine, and M3G were quantified by HPLC (see HPLC procedure).

Plasma and Urine M6G Kinetics. One day before experiment, rats were anesthetized with chloral hydrate (300 mg/kg, ip) and the femoral artery was cannulated with PE-50 (Biotrol, Paris). M6G (80.4 μmol/kg) dissolved in water was administered by gavage at a volume of 4.5 ml/kg. 300 μl of blood sample was collected from each rat (N = 6) at 0.08, 0.17, 0.25, 0.5, 1, 2, 3, 5, and 24 hr after drug administration and then centrifuged at 3000 rpm for 5 min to collect plasma. Urine specimens from the six rats were collected over a 2-hr period during the first 8 hr and from 8 to 24 hr. Plasma and urine samples were stored at −20°C until morphine, M3G, and M6G assays.

HPLC Procedure. Concentrations of M6G, M3G, and morphine were determined by reversed phase high pressure liquid chromatography as previously described by Déchélette et al. (1993) and D’Honneur et al. (1994). Retention times of M3G, M6G, morphine, and hydromorphone (internal standard) were 5.7, 8.1, 11.3, and 15.7 min, respectively. The limit of quantitation of M3G, M6G, and morphine was 0.009, 0.012, and 0.003 nmol/ml, respectively, with 100 μl of sample injected (coefficient of variation <20%). The intra- and interday reproducibility of at least 10 replicate samples were, respectively, 4.5% and 9.7% for M6G, 2.8% and 8.6% for M3G, and 6.0% and 11.2% for morphine.

Kinetic and Statistical Analysis. All data are expressed as mean ± SD. Statistical significance was set at p < 0.05 for the acidic hydrolysis using the two-way ANOVA and for the intestinal metabolism using Student’s t test (GraphPad Prism, San Diego).

Theoretical considerations for the recirculating perfusion system have been described by Pang and Gillette (1978). The perfusate concentration-time curve was fitted to one-compartment open model using nonlinear regression by extended least squares analysis (Siphar, Simed, Créteil, France). Pharmacokinetic parameters were calculated by fitting the data to a monoeponential equation (C = C0 × e−ke t), where C0 is the extrapolated concentration at t = 0 and ke the elimination rate constant. The corresponding half-time (min) is calculated as 0.693/ke. The apparent hepatic uptake clearance (CLu) was obtained by multiplying ke by the volume of distribution (V = dose/AUC0–∞ × ke), where the area under the curve from zero to infinity (AUC0–∞) was calculated as AUC0–∞ = C0/ke. The hepatic ratio (E) was calculated as CLu/Q, wherein Q is the perfusate flow rate (ml/min). The % of morphine or M3G recovered in perfusate and bile was calculated as the morphine/M6G or M3G/M6G AUC1–∞ ratios, where AUC1–∞ was calculated by using the linear trapezoidal method.

The amount of M6G excreted in each bile sample was calculated by multiplying the sample volume by concentration. The cumulative amount of M6G at time t(B) and t(B) were calculated from these values. Pharmacokinetic analysis was conducted by plotting the amount remaining to be excreted (B0 – B) vs. time (t) on a semilogarithmic scale, according to the equation: 

\[ B_0 - B = B_e \times e^{-k_{el}t} \]

where \( B_e \) was the biliary elimination rate constant. The corresponding t1/2 was calculated as 0.693/ke. The value of B0 was estimated by the rectangular hyperbola equation (GraphPad Prism, San Diego).

The area under the M6G, M3G, and morphine plasma and urine concentration-time curves from 0 to the last measured time (AUC0–∞) was calculated by using the linear trapezoidal method.

Results and Discussion

Acidic and enzymatic hydrolysis of M6G was studied first. Acidic hydrolysis of morphine glucuronides is currently used to assess morphine in urine drug-testing laboratories, but under drastic conditions: 1 hr in a boiling water bath with concentrated HCl. Moreover, M6G is not so readily hydrolyzed as M3G (Romberg and Lee, 1995). Acid hydrolysis must be studied because the first organ entered by an orally administered drug is the stomach. We investigated the stability of...
M6G in acidic medium at 37°C for 30 min or 1 hr at pH ranging from 1 to 5. Recovery of morphine after incubation of M6G (2.01 μmol/liter) in acidic solution is shown in fig. 1. At pH 1 and 2, hydrolysis of M6G was significantly higher (p < 0.05) after 1 hr of incubation than after 30 min. At pH 3 to 5, there was no significant difference (p > 0.05) between values at 30 min and 1 hr. The per cent of morphine recovery was significantly higher at pH 3 (both incubation times) and pH 2 (1 hr of incubation) than at the other pH. The maximum per cent of morphine recovered in solution reached 4.1 ± 0.2% at pH 3.

The major route of M6G hydrolysis to morphine could therefore be via the action of β-glucuronidase. This is why the metabolism of M6G was investigated in medium likely to express β-glucuronidase activity, i.e. in intestinal feces. The per cent of morphine formation resulting from M6G (2.01 μmol/liter) incubation with total pooled intestinal feces and with the reference medium containing β-glucuronidase at pH 5.2 was found stable between 17 and 23 hr (initial experiment). Using this last time value, morphine formation was measured after incubating 2.01 μmol/liter of M6G in feces from different parts of the intestine (fig. 2). In the small intestine, the formation of morphine was minor (less than 2%). When M6G was incubated in colon feces, morphine formation represented 85.6 ± 12.9% of the initial concentration of M6G. This value is significantly higher than that obtained following M6G incubation with the reference medium containing β-glucuronidase at pH 5.2 (p < 0.05). These results confirm the bacterial origin of the β-glucuronidase activity described by several authors (Hawskworth et al., 1971; Koster et al., 1985). The increase in M6G hydrolysis in the large intestine is consistent with the increasing colonization of the distal intestine by enterobacteria. Hawskworth et al. (1971) demonstrated that the strict anaerobes, bacteroides, and bifidobacteria are probably responsible for most of the β-glucuronidase activity in the large intestine. We can conclude from our results that the intestinal hydrolysis of M6G is high in feces from the colon. This phenomenon of M6G hydrolysis in the large intestine of rats would be much less marked in humans because of the very low β-glucuronidase activity (1500-fold lower than in rats) (Hawskworth et al., 1971).

Previous studies have demonstrated that unchanged morphine and its metabolites are extracted by hepatocytes and diffuse back into the circulation across sinusoidal membranes of the kidney (Hasselstrom and Sawe, 1993; Osborne et al., 1992) and are excreted in urine. They can also be excreted via bile as morphine and as the glucuronide metabolite (50–55%) before being reabsorbed as morphine following cleavage within the gastrointestinal tract. The role of hepatic clearance on M6G was investigated by using the IPRL model. Per fusate concentration-time profiles for M6G, M3G, and morphine in the IPRL model are presented in fig. 3. Per fusate M6G concentrations declined monoe xponentially (fig. 3, inset) with a terminal half-life of 66.4 ± 20.6 min. Hydrolysis of M6G was low with, respectively, 7.7 ± 4.5 and 4.7 ± 3.6% of the initial concentration recovered as morphine and M3G in the perfusate samples at 180 min. This low amount of morphine resulting from M6G hydrolysis can be explained by the low activity of β-glucuronidases in liver or by its conversion into M3G, which has been shown in the rat to be the main metabolite accounting for 73% of eliminated morphine (Evans and Shanahan, 1995). The low M6G hydrolysis was partially confirmed by the finding of less than 5% of M3G in the perfusate. These data were also in agreement with the low biliary excretion (0.03%) of morphine and therefore the high level (88.7 ± 11.2% of the dose) of M6G recovered in bile at 1 hr as shown in fig. 4. Pharmacokinetic parameters describing the disposition of M6G in IPRL are given in tables 1 and 2. The hepatic clearance (2.25 ± 1.1 ml/min) and hepatic extraction ratio (0.04 ± 0.02) of M6G were very much lower than those reported for morphine (25.9 ± 1.1 ml/min and 0.86 ± 0.037, respectively) (Evans and Shanahan, 1995). The lower hepatic extraction ratio of M6G is logical considering that a glucuronide is a metabolic end product. The elimination rate constant of M6G in the bile (0.04 ± 0.005 min⁻¹) is faster than that of the perfusate (0.012 ± 0.005 min⁻¹). This suggests an active process of elimination in the bile as has been described for bilirubin glucuronide and some conjugated drugs (Kramer and Wess, 1996). M6G has been described as a substrate for P-glycoprotein (Huwyler et al., 1996) and could be actively excreted in the bile via a carrier-mediated transport system (Polt et al., 1994; Van Crugten et al., 1991).

The high excretion of M6G in bile raises the question of the contribution of M6G to the EHR of morphine. Glucuronides were assumed to be absorbed poorly per se in the last gut compartment but hydrolyzed by intestinal β-glucuronidase to liberate morphine, which is absorbed within the gut. A model of M3G EHR was developed by Ouellet and Pollack (1995) to understand the influence of M3G disposition on morphine pharmacokinetics. They demonstrated that 20% of M3G is excreted in bile after M3G administration and that the remainder of the dose is recovered in urine. No morphine was detected in serum, but, following prolonged exposure to M3G, Ekblom et al. (1993) detected a maximum plasma morphine concentration of 0.15 nmol/ml. The contribution of M6G to EHR of morphine is probably less marked than that of M3G because of the greater chemical stability of M6G; the nature of the chemical bond between morphine and glucuronic acid implies that M6G (alcoholic position) is less prone to hydrolysis than M3G (phenolic position) (Romberg and Lee, 1995).

This contribution of M6G to morphine recirculation was estimated by the in vivo experiment where M6G was orally administered. The
M3G was not detectable. Vertical bars represent the mean ± SD of four experiments.

**TABLE 1**

**Pharmacokinetic parameters of hepatic uptake of M6G, morphine, and M3G in IPRL calculated from perfusate data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>M6G</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_e$ (min$^{-1}$)</td>
<td>0.012 (0.005)</td>
<td>Morphine 7.71 (4.55)</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>66.4 (20.6)</td>
<td>M3G 4.71 (3.60)</td>
</tr>
<tr>
<td>V (ml)</td>
<td>200 (96.4)</td>
<td></td>
</tr>
<tr>
<td>$V_{h}$ (ml/min)</td>
<td>2.25 (1.1)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.04 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2**

**Pharmacokinetic parameters of M6G in IPRL calculated from biliary data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_e$ (min$^{-1}$)</td>
<td>0.04 ± 0.005</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>18.6 ± 2.5</td>
</tr>
<tr>
<td>Morphine recovery (%)</td>
<td>0.03 ± 0.005</td>
</tr>
<tr>
<td>M3G recovery (%)</td>
<td>ND</td>
</tr>
</tbody>
</table>

plot of M6G, M3G, and morphine plasma concentrations vs. time after oral administration of M6G (80.4 μmol/kg) is shown in fig. 5. Five minutes after M6G administration, M6G was detected in plasma. The systemic absorption of M6G was slow as the peak level was observed 8 hr after M6G administration. A detectable level of morphine and M3G was, respectively, found 2 and 3 hr after M6G administration, peaked at 7 hr, and remained stable up to the last experimental time. Based upon the comparison of the respective AUC$ _{0-24}$, we found that morphine and M3G represented 10.5 ± 4.3% and 12.9 ± 5.1% of M6G, respectively. The delay before appearance of morphine and M3G in plasma demonstrates that, in agreement with our previous findings on the elevated hydrolysis of M6G in the colon, M6G molecules have to reach the distal portion of the intestine to be hydrolyzed into morphine. This time delay is compatible with the 3–6 hr transit time that is required by M3G to reach the cecum after its biliary excretion (Walsh and Levine, 1975). Another finding is the absence of a significant lag-time in the absorption of M6G, which is detected in plasma as soon as 5 min after administration. However, the absorption process was prolonged over the 24-hr experiment probably due to a dual mechanism: 1) a first-pass absorption of M6G *per se* while still in the proximal intestine and 2) a second absorption phase resulting from the biliary excretion of M6G and its reabsorption. This *in vivo* experiment also revealed that the extent of M6G absorption is low; at 24 hr, the percentage of M6G, morphine, and M3G excreted in urine after oral M6G administration was only 3.2 ± 1.1%, 0.8 ± 0.3%, and 0.7 ± 0.3%, respectively. This conclusion is supported by a previous experiment where we found 47.8 ± 13.9% of the M6G dose in urine after ip administration of M6G (unpublished data). Assuming a complete absorption of M6G after ip, we can assume that the bioavailability of oral M6G does not exceed 5–10%. These urine data can be considered as reliable according to the available information on the renal disposition of morphine and glucuronide conjugates in a rat isolated perfused kidney model (Van Crugten et al., 1991). After M6G administration, no morphine was detected in urine or perfusate, indicating that no deconjugation occurs in the rat kidney. More, our results showed that the morphine/M6G plasma AUC$ _{0-24}$ ratio is 3.35 times higher than the morphine/M6G urinary cumulative amount ratio at 24 hr, suggesting that the urinary handling of these two compounds is different. Morphine undergoes active reabsorption in addition to glomerular filtration and active tubular secretion in the rat kidney (Nation et al., 1996; Van Crugten et al., 1991). M3G is predominantly filtered with little reabsorption, whereas M6G is largely reabsorbed by the nephron.

The present study clearly demonstrates that M6G is poorly metabolized, unlike morphine, which is 90% metabolized. This is in agreement with previous studies which indicate that M3G, the main metabolite of morphine (Ouellet and Pollack, 1995), is also poorly metabolized in rat. Lötisch et al. (1996) have also reported that neither morphine nor M3G are detected in human plasma after iv administration of M6G.

Several studies have demonstrated that M6G is a more potent analgesic agent than morphine (Pasternak et al., 1987; Paul et al., 1989; Stain et al., 1995). Our study focused on the low deconjugation of M6G *in vitro* and *in vivo*, except in the colon, which confirms that the analgesic properties of M6G are not due to its biotransformation.
into morphine. However, the low oral bioavailability of M6G could limit the interest of this route of administration, but the slowness of its absorption over several hours could represent a type of physiological slow-release system useful for prolonging the analgesic effects of M6G.

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References